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### Authors

Chen, Jingxun  
McSwiggen, David  
Ünal, Elçin

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Video Article

# Single Molecule Fluorescence *In Situ* Hybridization (smFISH) Analysis in Budding Yeast Vegetative Growth and Meiosis

Jingxun Chen<sup>1</sup>, David McSwiggen<sup>2</sup>, Elçin Ünal<sup>1</sup>

<sup>1</sup>Department of Molecular and Cell Biology, Barker Hall, University of California, Berkeley

<sup>2</sup>Department of Molecular and Cell Biology, Li Ka Shing Center, University of California, Berkeley

Correspondence to: Elçin Ünal at [elcin@berkeley.edu](mailto:elcin@berkeley.edu)

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## Abstract

Single molecule fluorescence *in situ* hybridization (smFISH) is a powerful technique to study gene expression in single cells due to its ability to detect and count individual RNA molecules. Complementary to deep sequencing-based methods, smFISH provides information about the cell-to-cell variation in transcript abundance and the subcellular localization of a given RNA. Recently, we have used smFISH to study the expression of the gene *NDC80* during meiosis in budding yeast, in which two transcript isoforms exist and the short transcript isoform has its entire sequence shared with the long isoform. To confidently identify each transcript isoform, we optimized known smFISH protocols and obtained high consistency and quality of smFISH data for the samples acquired during budding yeast meiosis. Here, we describe this optimized protocol, the criteria that we use to determine whether high quality of smFISH data is obtained, and some tips for implementing this protocol in other yeast strains and growth conditions.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/57774/>

## Introduction

Dynamic regulation of gene expression drives the development of an organism, as well as its response to environmental stress, infection, and changes in metabolism. Studies that focus on transcriptional regulation rely on technologies that measure RNA abundance. One such method, termed single molecule fluorescence *in situ* hybridization (smFISH), is used for detection of individual RNA molecules in single cells<sup>1,2</sup>. This method allows measurement of the cell-to-cell variability in gene expression and determination of intracellular RNA localization.

In the most commonly used smFISH technique, detection of an individual RNA molecule requires multiple short DNA probes (often ~48 20-mer probes) that are complementary to the target RNA and are conjugated to the same fluorescent dye. Binding of a single fluorescent probe results in weak signal, but the signal from the ensemble of all the probes is robust. This feature greatly improves the signal-to-noise ratio because even though a single probe can exhibit off-target binding, such signal is expected to be very weak compared to that of the target RNA molecule<sup>3</sup>. Within error of detection, the number of RNA molecules can be counted and compared across various growth conditions and among different mutants.

Since its first development, smFISH has been adapted to study various aspects of gene expression<sup>4</sup>, such as transcription elongation<sup>1,2</sup>, splicing<sup>5,6</sup>, transcriptional bursting<sup>7,8,9</sup>, intracellular allelic expression<sup>10,11,12</sup>, and RNA localization<sup>13,14,15</sup>. Recently, we have used this method to study the expression of two transcript isoforms of the same gene, in which the short transcript isoform (*NDC80*<sup>ORF</sup>) has its entire sequence shared with the long isoform (*NDC80*<sup>uti</sup>)<sup>16</sup>. To uniquely identify the two mRNA isoforms, we used two probe sets: one set is specific to the unique sequence of *NDC80*<sup>uti</sup>, and the other set, conjugated to another fluorescent dye, binds to the common region of the two isoforms. The *NDC80*<sup>uti</sup> RNA is identified as a colocalized spot with both fluorescent signals, whereas the *NDC80*<sup>ORF</sup> RNA is the one that contains only the signal from the common probe set. Since the number of the *NDC80*<sup>ORF</sup> transcripts is calculated by a "subtraction" method, high efficiency of probe hybridization and a high signal-to-noise ratio are necessary to confidently identify smFISH spots and reduce error propagation.

This paper describes an optimized protocol for performing smFISH in the budding yeast *Saccharomyces cerevisiae*. In this protocol, the cell number, fixation time, digestion time, digestion buffer, probe concentration, and hybridization buffer used for the smFISH experiments were optimized for the SK1 strain background of *S. cerevisiae* undergoing vegetative growth or meiosis. However, we also note in this manuscript: (1) the method to check the quality of smFISH data after image acquisition, and (2) the steps in the protocol that may require additional optimization for different strain backgrounds and growth conditions.

## Protocol

NOTE: All the buffers and the media used in this protocol are listed in **Table 1**. The vendor information for the reagents is listed in the **Table of Materials**.

Day 1/Day 1-2:

NOTE: For vegetative culture, grow cells to an OD<sub>600</sub> of 0.4 to 0.6 in a preferred medium. For meiotic culture, induce cells to undergo meiosis using a preferred method (typically culturing in an OD<sub>600</sub> of 1.85).

## 1. Sample Fixation and Digestion

1. Fix a total of ~3.5 OD<sub>600</sub> of cells in 3% formaldehyde.
  1. For vegetative culture, add 5.52 mL of culture to 480 µL of 37% formaldehyde in 15-mL conical tubes.
  2. For meiotic culture, add 1840 µL of culture to 160 µL of 37% formaldehyde in 2-mL microcentrifuge tubes. Invert ~5 times to mix.  
CAUTION: Formaldehyde is toxic. Handle and dispose according to institutional regulations.
2. Place tubes on a roller drum at room temperature for 20 min. For meiotic samples, after fixing at room temperature for 20 min, continue fixing overnight at 4 °C, rotating.  
NOTE: The overnight fixation increases the reproducibility and quality of the smFISH data obtained for meiotic samples. Fixation time should be optimized.
3. While the samples are fixing, thaw 200 mM Vanadyl Ribonucleoside Complexes (VRC) at 65 °C for at least 10 min.
4. Prepare digestion master mix in a 15-mL tube: for 1 sample, mix 425 µL of Buffer B with 40 µL of 200 mM VRC (warmed to 65 °C); for 5 samples, mix 2125 µL of Buffer B with 200 µL of 200 mM VRC (warmed to 65 °C). Vortex ~5 s to fully resuspend the VRC before adding to the master mix, which will appear light brownish green after VRC addition.  
NOTE: Addition of VRC during digestion improves the consistency of smFISH results, potentially by inhibiting the nuclease contaminant introduced by the zymolyase mixture, which is purified from crude extract. The amount of VRC required at this step should be optimized.
5. For vegetative samples, centrifuge the tubes at ~1057 x g for 3 min. For meiotic samples (after overnight fixation), centrifuge at 21,000 x g for 1.5 min. Decant or aspirate the supernatant to formaldehyde waste.  
NOTE: All the centrifugation steps are performed at room temperature.
6. Resuspend cells in 1.5 mL of cold Buffer B by pipetting up and down or by inverting the tubes and flicking to mix. Transfer vegetative samples to 2-mL tubes after resuspension.
7. Centrifuge at 21,000 x g for 1.5 min. Remove the bulk of the liquid by vacuum aspiration or by pipetting, leaving behind ~100 µL.
8. Resuspend the cells in 1.5 mL of cold Buffer B.
9. Centrifuge at 21,000 x g for 1.5 min. Remove the bulk of the liquid by vacuum aspiration or by pipetting, leaving behind ~100 µL.
10. Resuspend cells in 1.5 mL of cold Buffer B. Centrifuge at 21,000 x g for 1.5 min. Aspirate the liquid completely by vacuum or by pipetting.
11. Resuspend cells in 425 µL of digestion master mix, and briefly vortex to resuspend. Add 5 µL of 100T 10 mg/mL zymolyase to each tube.  
NOTE: Vortex the zymolyase every time before adding to the tubes. Add zymolyase to each tube individually, instead of adding to the master mix. Both steps help maintain digestion consistency among tubes because zymolyase precipitates quickly.
12. Vortex 2-3 s to mix. Place the tubes on a roller drum, and digest at 30 °C for 15-30 min. For vegetative cells and early meiotic stages, it usually takes ~15 min, and for meiotic prophase and meiotic divisions, it usually takes ~30 min.  
NOTE: Check on the microscope every 5 min after 15 min, and stop the digestion when ~80% of cells appear non-transparent and non-refractive. **From this point on, cells are very fragile. Handle cells gently and avoid using vacuum aspiration or vortexing.**
13. Centrifuge the tubes at ~376 x g for 3 min. Remove the liquid completely by pipetting.
14. Gently resuspend cells with 1 mL of Buffer B by pipetting up and down 1-2 times to mix.
15. Centrifuge the tubes at ~376 x g for 3 min. Remove Buffer B liquid by pipetting. Gently resuspend cells in 1 mL of 70% ethanol (diluted with RNase-free water).
16. Incubate at room temperature for 3.5-4 hours.

## 2. Hybridization

1. Bring formamide to room temperature (for 50-mL aliquot, it takes ~30 min in water bath).  
NOTE: Do not open the formamide bottle until the bottle reaches room temperature to avoid oxidation of formamide.  
CAUTION: Formamide is toxic. Handle and dispose according to institutional regulations.
2. Prepare 10% formamide wash buffer in a 15-mL conical tube.
3. Centrifuge the tubes at ~376 x g for 3 min. Remove 500 µL of 70% ethanol by pipetting. Gently pipet up and down to resuspend the remaining cells, and then transfer the cells to low-adhesion tubes.  
NOTE: Using low-adhesion tubes greatly reduces cell loss during subsequent washes.
4. Centrifuge the tubes again at ~376 x g for 3 min. Remove all the ethanol by pipetting.
5. Add 1 mL of 10% formamide wash buffer, and gently pipet up and down 2-3 times to resuspend the cells.
6. Allow the cells to sit at room temperature for ~20 min while preparing the Hybridization Solution. For 1 sample, mix 50 µL of Hybridization Buffer (room temp.), 5 µL of 200 mM VRC (warmed to 65 °C), and 1 µL of each probe (200 nM final). For 5 samples, mix 250 µL of Hybridization Buffer (room temp.), 25 µL of 200 mM VRC (warmed to 65 °C), and 5 µL of each probe (200 nM final).
  1. Thaw Hybridization Buffer (frozen at -20 °C) to room temperature before opening the tube to prevent formamide oxidation.
  2. After adding the 200 mM VRC, vortex for 5-10 s before adding the probes, which are designed and purchased commercially, and reconstituted according to the manufacturer's instructions.

3. If two probes are co-incubated, add 1  $\mu$ L of the 1:10 dilution of the probe #1 stock, as well as 1  $\mu$ L of the 1:10 dilution of the probe #2 stock, to make a final dilution of ~1:500 for either probe. The concentration needed for the best signal-to-noise ratio needs to be optimized (See Discussion for details).
7. Centrifuge the samples at ~376 x g for 3 min. Remove the supernatant into hazardous waste by pipetting.
8. Add at least 50  $\mu$ L of Hybridization Solution to each tube. Flick the tubes to mix.
9. Incubate at 30 °C on a roller drum for at least 16 hours in the dark.

Day 2/Day 3

### 3. Washing and imaging

1. Bring formamide to room temperature.
2. Prepare 10% formamide wash buffer (FWB) in a 15-mL conical tube.
3. Remove the tubes from the roller drum and place them into a foil-covered box to protect from light.
4. Centrifuge the samples at ~376 x g for 3 min. Remove the supernatant into hazardous waste by pipetting.
5. Resuspend in 1 mL of 10% FWB by gently pipetting up and down 2-3 times.
6. Incubate at 30 °C for 30 min (not rotating) in the foil-covered box.
7. Centrifuge the samples at ~376 x g for 3 min. Remove the supernatant to hazardous waste by pipetting, and leave ~50  $\mu$ L.
8. Meanwhile, prepare DAPI/FWB in a 15-mL conical tube: For 1 sample, mix 1000  $\mu$ L of 10% formamide wash buffer with 1  $\mu$ L of 5 mg/mL DAPI. For 10 samples, mix 10 mL of 10% formamide wash buffer with 10  $\mu$ L of 5 mg/mL DAPI. Resuspend in 1 mL of DAPI/FWB by gently pipetting up and down 2-3 times.
9. Incubate at 30 °C for 30 min (not rotating) in the foil-covered box.
10. Thaw anti-bleach reagents on ice.
11. Centrifuge the samples at ~376 x g for 3 min. Remove the supernatant completely by pipetting. If needed, centrifuge again to remove all of the supernatant.
12. For samples that are not imaged immediately, resuspend the pellet in 50  $\mu$ L of GLOX Buffer **without** enzymes. Pipet up and down 3-4 times to mix.
  1. Keep all the unimaged samples in the foil-covered box at 4 °C until ready to image. When ready to image, centrifuge the samples at ~376 x g for 2 min and remove the supernatant completely by pipetting. Resuspend in GLOX with enzymes as below.
13. For samples being imaged immediately, add 15-20  $\mu$ L of GLOX buffer with enzymes. Gently pipet up and down to mix.
 

NOTE: The volume added can vary depending on the size of the cell pellet. We recommend resuspending the pellet in 15  $\mu$ L of GLOX buffer with enzymes and check the cell density on microscope. If cells are too dense (cells clumping on top of one another), add extra GLOX buffer with enzymes. If cells are too sparse, centrifuge the sample and remove ~5  $\mu$ L of buffer.
14. Pipet 5  $\mu$ L onto a coverslip (18 mm x 18 mm, No. 1), and put the coverslip on a slide.
15. Put a laboratory wipe on top of the slide where the coverslip is placed. Gently press on the laboratory wipe to set slide (should see liquid coming off from all four edges of the coverslip).
16. Transfer the slide to the microscope room in a box covered by aluminum foil.
17. Image using a wide-field fluorescence microscope with high magnification (60-100X) and high numerical aperture.
 

NOTE: To collect the maximal number of photons emitted by the smFISH probes, confocal microscopes are not recommended, as they significantly limit the amount of light to be collected. Here, the data were collected on a microscope equipped with a 100X, 1.4 NA objective, using filters CY5 (EX632/22, EM679/34) imaged at 1.3 s, 100% T; TRITC (EX542/27, EM597/45), 1.3 s, 100% T; and DAPI (EX390/18, EM435/48), 0.05-0.1 s, 32%-50% T. A bright-field reference image should also be acquired. Acquire 15-25 slices with a step size of 0.1-0.2  $\mu$ m, from entirely below the focus of the field of view to entirely above, in order to ensure that all of the RNA spots are accounted for.

### 4. Image Analysis

1. Analyze the smFISH data with published smFISH analysis tools such as FISH-quant<sup>17</sup> and StarSearch<sup>2</sup>, or with custom-made programs, depending on the exact requirements of the experiment.
 

NOTE: A good analysis pipeline should allow the users to determine a threshold to separate the true RNA spots from the background, and output statistics such as the X, Y, and Z (optional) coordinates and the intensity of each spot, the number of spots in each cell, and possibly fitting parameters as a way to filter out false detections.

### Representative Results

To evaluate how well the smFISH protocol worked (outlined in **Figure 1**), we designed a set of 54 probes that tile the open reading frame of the *NDC80* gene (**Figure 2A**, top). The probe located at the most 5' end is referred to as Probe 1; the next one, Probe 2; and the third one, Probe 3, etc. The 27 probes assigned an odd number (Probe 1, 3, 5...) are all conjugated to the CAL Fluor 590 (CF590) fluorescent dye; and the 27 probes assigned an even number (Probe 2, 4, 6...), conjugated to the Quasar 670 (Q670) fluorescent dye. Hence, these alternating probe sets are often referred to as "odd/even" probes. Upon hybridization, both probe sets should label the same transcript.

After spot detection, we used a few measurements to judge the quality of the smFISH data set. The first one was the degree and quality of colocalization for the odd/even probes. In our case, 88% of all the smFISH spots colocalized (**Figure 2A and 2B**), with greater than 95% of the spots paired within 2 pixels of each other (**Figure 2C**, paired), which is within the expected value given any chromatic and detection aberration between the two fluorescent channels. By comparison, fewer than 10% of unpaired spots had a nearest-neighbor distance of less than 2 pixels, showing that the probability of misidentifying a spot pair is low (**Figure 2C**). The 12% of unpaired spots were evenly divided between the two channels (**Figure 2B**, compare CF590-only with Q670-only); and thus, we concluded that for this 2.4 kb gene with a range of expression between zero to 45 transcripts per cell, ~94% of RNA molecules were accurately detected in each fluorescent channel. If the smFISH protocol were suboptimal, one would observe (1) a greater fraction of the smFISH spots with only one of the two fluorescent signals (non-colocalized), and/or (2) cells with a very low signal-to-noise ratio or no signal at all.

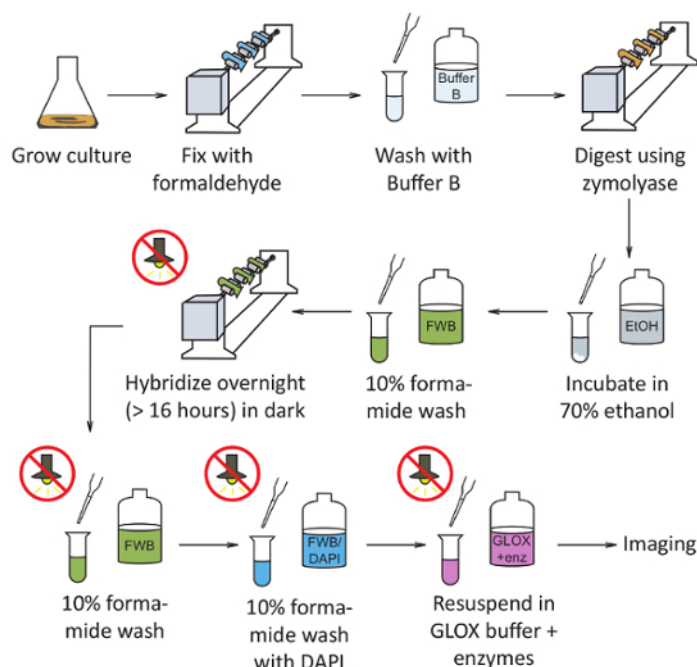
We next asked if the detection of the smFISH signal was biased with respect to the total number of RNA molecules per cell. In a population, the total number of a particular RNA in each cell lies in a distribution, with some cells harboring more RNA molecules than others. A good smFISH protocol should robustly detect the RNA regardless whether a high number or low number of RNA molecules is present in each cell. To test this, for each cell, we calculated the fraction of the smFISH spots with colocalized signals and the fraction with only one of the two fluorescent signals. After grouping the cells that had the same number of total spots per cell, we calculated the average fraction of colocalized (paired) or non-colocalized (CF590-only or Q670-only) spots in a given group, and graphed this average as a function of the total number of spots per cell (**Figure 3**). For each category of spots, the fractions were similar across the entire range of the total number of spots per cell. For instance, comparing the cells with a total of 20 fluorescent spots per cell versus those with a total of 30 spots, the average fractions of the colocalized spots were similar. This result suggested that our protocol could detect a range of RNA molecules in a cell (up to at least ~40 molecules per cell). If the protocol worked suboptimally, one might observe a bias. For example, the fraction of the spots with only one of the two fluorescent signals might increase as the total number of spots per cell increased.

Using this optimized protocol, we examined the expression of the *NDC80* gene, which encodes a kinetochore protein, in different growth conditions. The *NDC80* gene expresses two mRNA isoforms: The long undecoded transcript isoform, *NDC80<sup>luti</sup>*, has a ~400 base pairs extension at the 5' end in comparison to the short *NDC80<sup>ORF</sup>* isoform, but both transcripts share the coding region of the *NDC80* gene (See schematic in **Figure 4A**). We designed two sets of probes: The CF 590 set binds to the unique 5' region of *NDC80<sup>luti</sup>*; and the Q 670 set binds to the common region of *NDC80<sup>luti</sup>* and *NDC80<sup>ORF</sup>*. The *NDC80<sup>luti</sup>* transcripts were detected as the smFISH spots where the signals from both probe sets colocalized, whereas the *NDC80<sup>ORF</sup>* transcripts were those with the signal only from Q 670. Due to the size of the unique segment of *NDC80<sup>luti</sup>*, we could only tile 20 oligonucleotide probes along this region, which is fewer than the recommended 30 to 48 probes. Thus, we first determined if this probe set could robustly detect RNA in our optimized protocol. For either fluorescent channel, we graphed the signal-to-noise ratio (SNR, defined as the variance of the pixels surrounding a spot relative to the spot intensity) against the signal detected for each smFISH spot, and generated a scatterplot for all the spots identified in the entire field of view (example images in **Figure 4A** and plots in **Figure 4B**). Two distinct populations were clearly identified for both the Q 670 and the CF 590 probe sets (Optimized, **Figure 4B**), suggesting that the true smFISH spots (inside the grey area) could be separated from the background signal. Note that in the suboptimal condition, such separation was less obvious (Suboptimal, **Figure 4B**).

We used these two probe sets to study the expression of *NDC80<sup>luti</sup>* and *NDC80<sup>ORF</sup>* during vegetative growth and meiosis. In vegetative cells, robust signal from the Q 670 probe set was detected, but not from the CF 590 probe set (**Figure 5A**, vegetative), agreeing with the observation by northern blotting that only the short isoform was expressed during vegetative growth<sup>16</sup>. This result also suggested that the CF 590 probe set is specific, yielding low background in our optimized smFISH protocol. In contrast, robust signal from both probe sets was detected in meiotic prophase, and the majority of the spots had colocalized signal (**Figure 5A**, meiotic prophase). Together with northern blot analysis (**Figure 5B**), this observation confirmed that the long isoform *NDC80<sup>luti</sup>* was expressed specifically in meiosis. These two types of mRNAs were detected in the cytoplasm (outside of the DAPI region), suggesting that both were exported from the nucleus, consistent with the ribosome profiling data<sup>18</sup>.

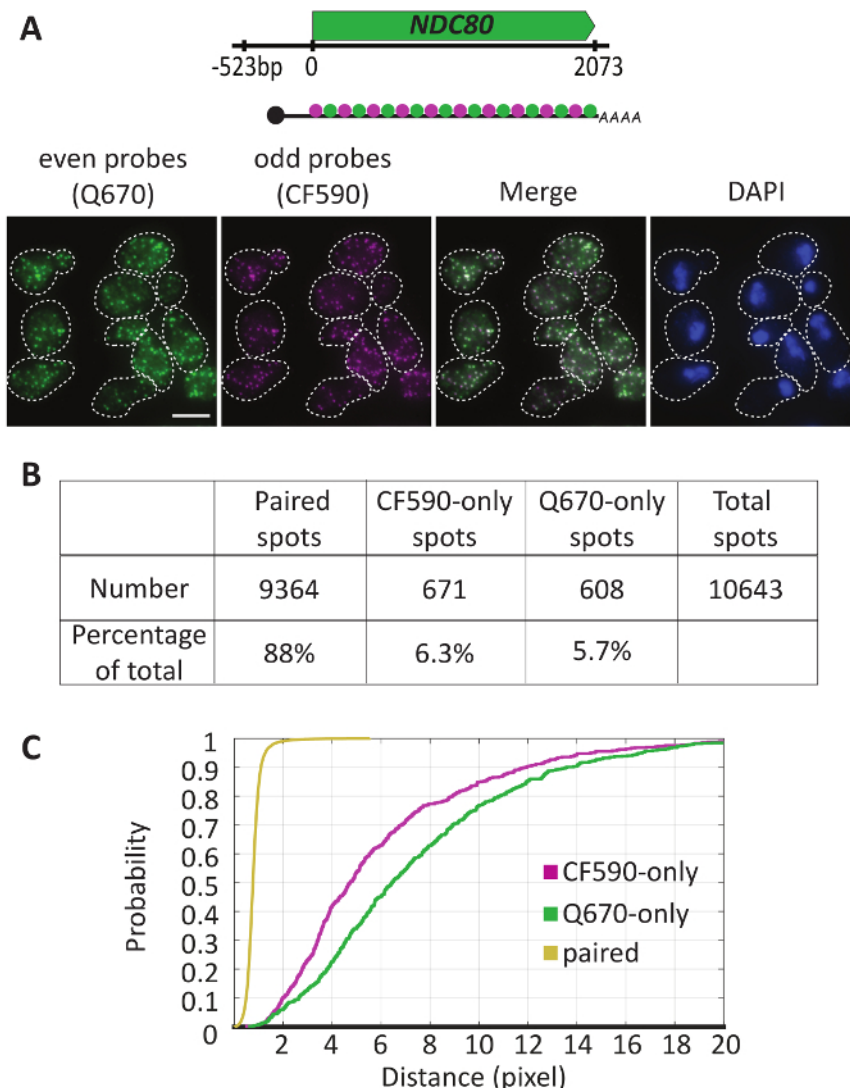
To determine if sufficient data were collected to accurately account for the biological variation intrinsic to our data set, we performed bootstrap analysis using the statistics of each cell, which included (1) the fraction of the colocalized spots per cell and (2) the fraction of the spots with one of the two fluorescent signals (CF 590 only and Q 670 only). In this analysis, a program randomly selected one cell from the 437 quantified cells for 500 iterations. Next, the mean and the variance of the respective statistics associated with these 500 selected cells were calculated. This process was then repeated for randomly selecting two cells from all the cells, without replacement; and then for randomly selecting three cells, etc. until one half of the total data set size was reached. The variance in the data set plateaued after ~40 cells, suggesting that after this point most of the variation in the mean is intrinsic to the data rather than an artifact of undersampling (**Figure 6**, inset). This effect became more obvious when we graphed the sample variance divided by the sample mean, as a function of the number of cells sampled in each iteration cycle (**Figure 6**). With the data shown, the change in variance became diminishingly small after ~60 cells. The number of cells quantified in an smFISH experiment should exceed the minimum number of cells required to achieve a stable mean and a plateau of the variance. In our case, we quantified over 95 cells per sample, per replicate. The total number of cells (> 400 cells) well exceeded the minimum number of cells (~60 cells) required to reflect the population mean.

With a custom-made Matlab program<sup>16</sup>, vegetative cells were found to have a median of 5 *NDC80<sup>ORF</sup>* transcripts per cell, whereas in meiotic prophase cells, the median significantly dropped to 4 transcripts per cell (two-tailed Wilcoxon Rank Sum test,  $p = 0.026$ ) (**Figure 7**). The median number of *NDC80<sup>luti</sup>* transcripts per cell was 21 transcripts, and 100% of the cells expressed the *NDC80<sup>luti</sup>* transcripts. We graphed the fraction of the cells with a given number of transcripts as a step-wise, relative frequency histogram because the number of mRNA molecules is a discrete quantity. The largest bin of each histogram was normalized to the same height.

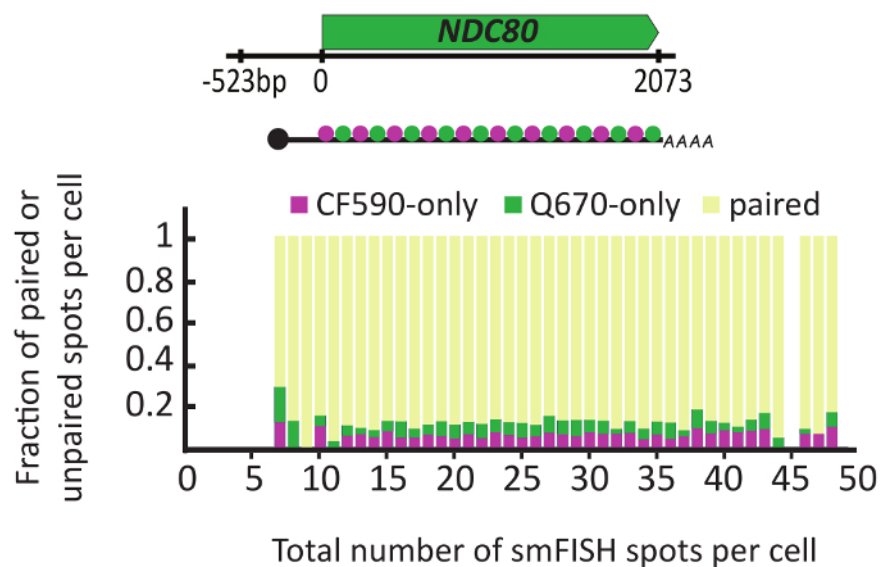


**Figure 1: Flow-chart for the smFISH protocol.** Cells are fixed with formaldehyde at room temperature for 20 min or at 4 °C overnight, for vegetative growth samples and meiotic samples, respectively. After washing in Buffer B three times, cells are digested by zymolyase until ~70% - 90% of cells are digested. The digested samples are then washed one time with Buffer B to remove the zymolyase, and subsequently permeabilized in 70% ethanol (EtOH) for ~3.5 hours. To prepare for hybridization, samples are first incubated in 10% formamide wash buffer (FWB) for ~20 min. Next, the samples are resuspended in ~50 µL of Hybridization Solution, which contains the fluorescent probes, for overnight incubation in the dark at 30 °C. After hybridization, the samples are incubated in 10% FWB for 30 min to wash away the excess probes, and then incubated in 10% FWB with 4',6-diamidino-2-phenylindole (DAPI) to stain the DNA. For the sample imaged immediately after the FWB/DAPI incubation, the sample is resuspended in the GLOX buffer supplemented with catalase, Trolox, and glucose oxidase (GLOX + enz); whereas, the other samples are resuspended in the GLOX buffer without enzymes, and stored at 4 °C up to ~3 hours. [Please click here to view a larger version of this figure.](#)



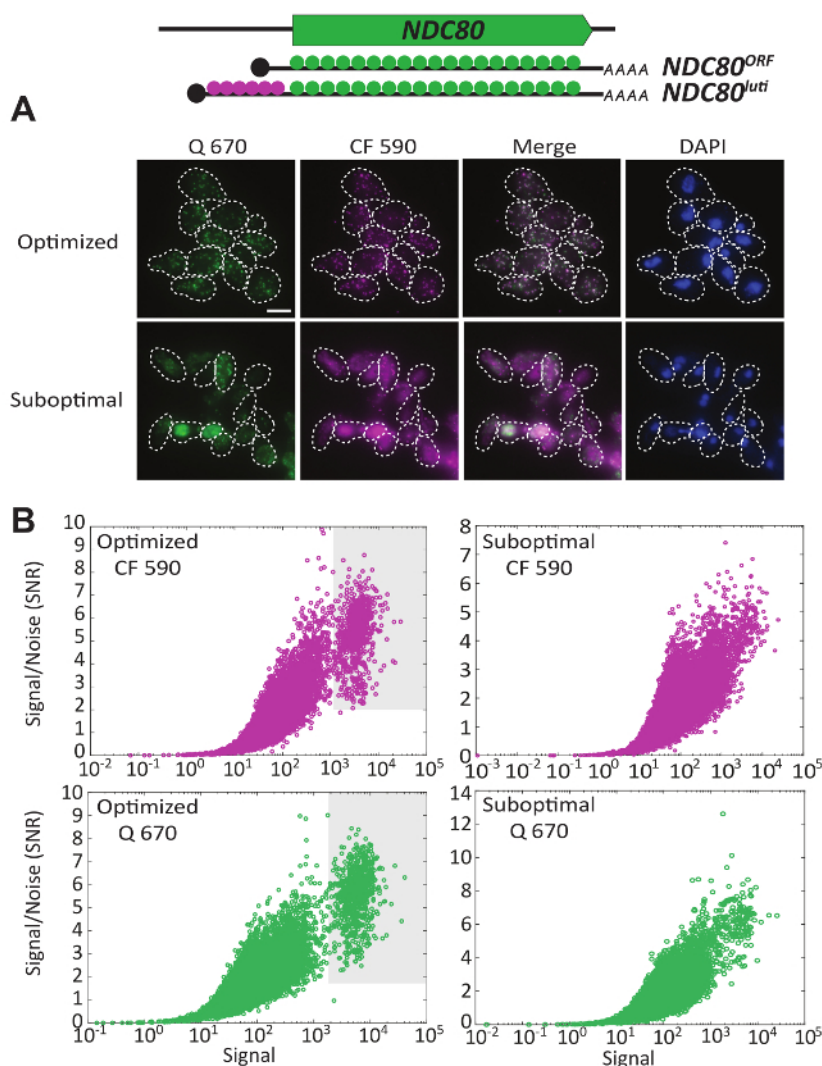


**Figure 2: Assessment of the smFISH quality using odd/even probes.** (A) Top: Schematic for the odd/even probe sets. Fifty-four oligonucleotide probes tiling the *NDC80* gene were designed. The odd-numbered probes were labeled with one fluorophore (CF590, shown in magenta), and the even-numbered probes, with another fluorophore (Q670, shown in green). Bottom: Representative smFISH images of meiotic prophase cells acquired using the odd/even probe sets. Samples were taken 6 hours after cells (UB8144) were transferred to sporulation medium, a time when these cells were arrested in meiotic prophase. DNA was stained with DAPI (shown in blue). Images are shown as the maximum-intensity projections of z-stacks. Scale bar: 5  $\mu$ m. (B) Percentage of the paired or unpaired smFISH spots obtained using the odd/even probe sets. A total of 428 meiotic prophase cells were analyzed, pooling from two independent experiments. This figure is modified from **Figure 2-figure supplement 4** of Chen *et al.*<sup>16</sup> (C) A Cumulative Density Function (CDF) of the distance between each pair of paired spots and distance between the nearest neighbor of an unpaired spot. For each detected spot in one fluorescent channel, the "k nearest neighbor" algorithm was applied to identify the closest detected spot—and the distance to that spot—in the complementary channel. Localizations that were mutual nearest neighbors were considered to be paired, and a new list was generated recording the paired, CF590-only, and Q670-only detections. For each category of detections, a CDF histogram of the distances was plotted in Matlab, confirming that correctly paired spots indeed were much closer in distance than those without a corresponding spot in the other channel. [Please click here to view a larger version of this figure.](#)

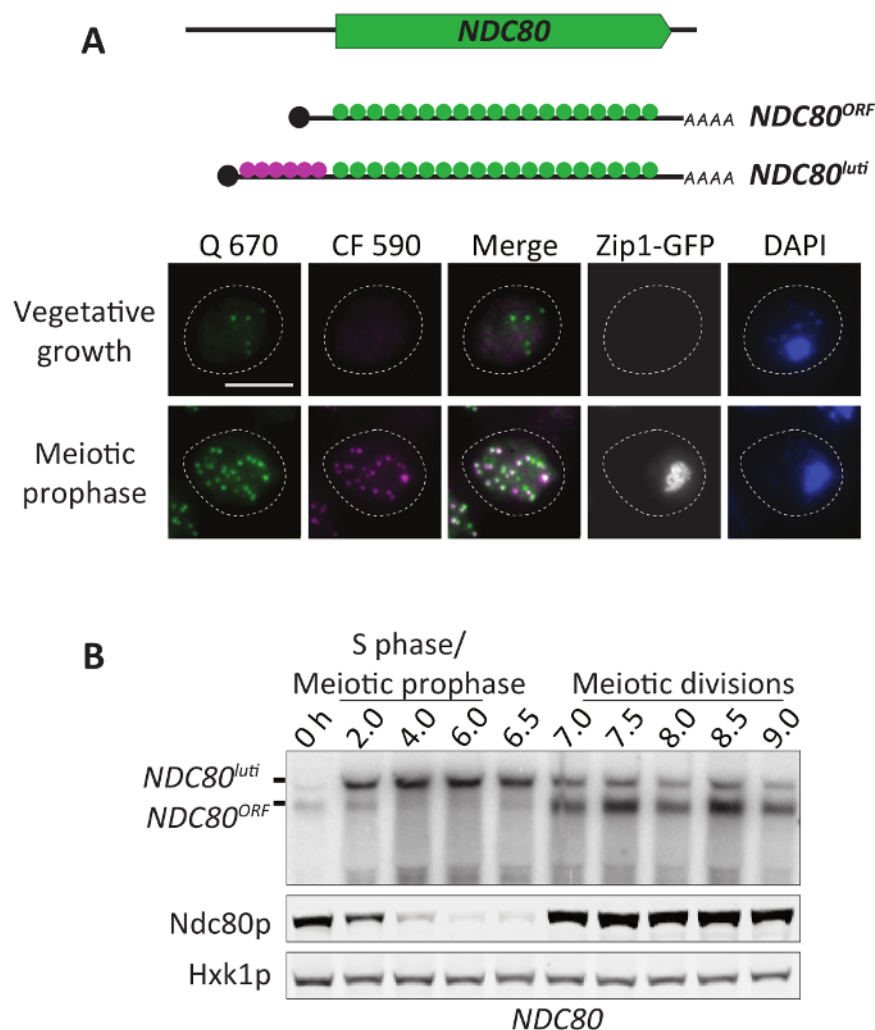


**Figure 3: Fractions of paired and unpaired spots, as a function of the total RNA number per cell.** To test if the detection and pairing of the smFISH spots was biased at different expression levels, individual cells were grouped by total RNA expression. The mean fractions of paired, CF590-only, and Q670-only detections were calculated for each group of cells. [Please click here to view a larger version of this figure.](#)

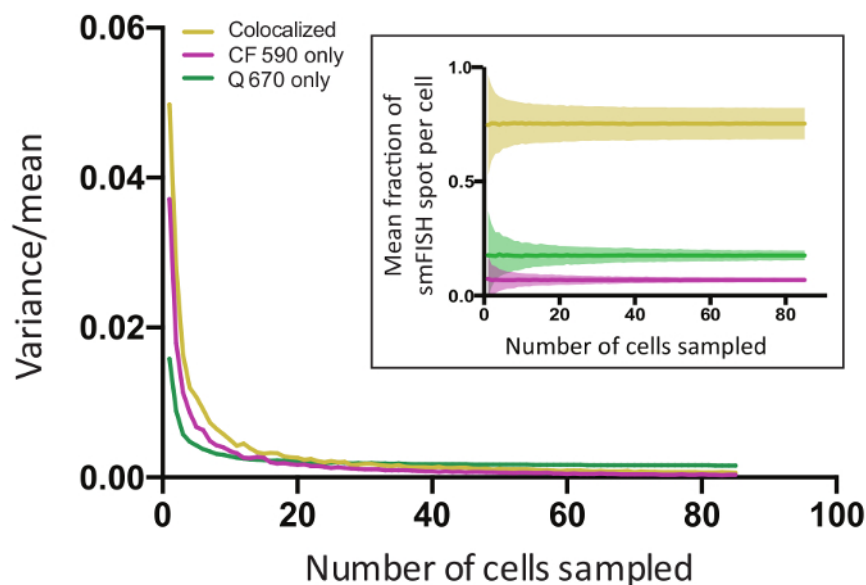




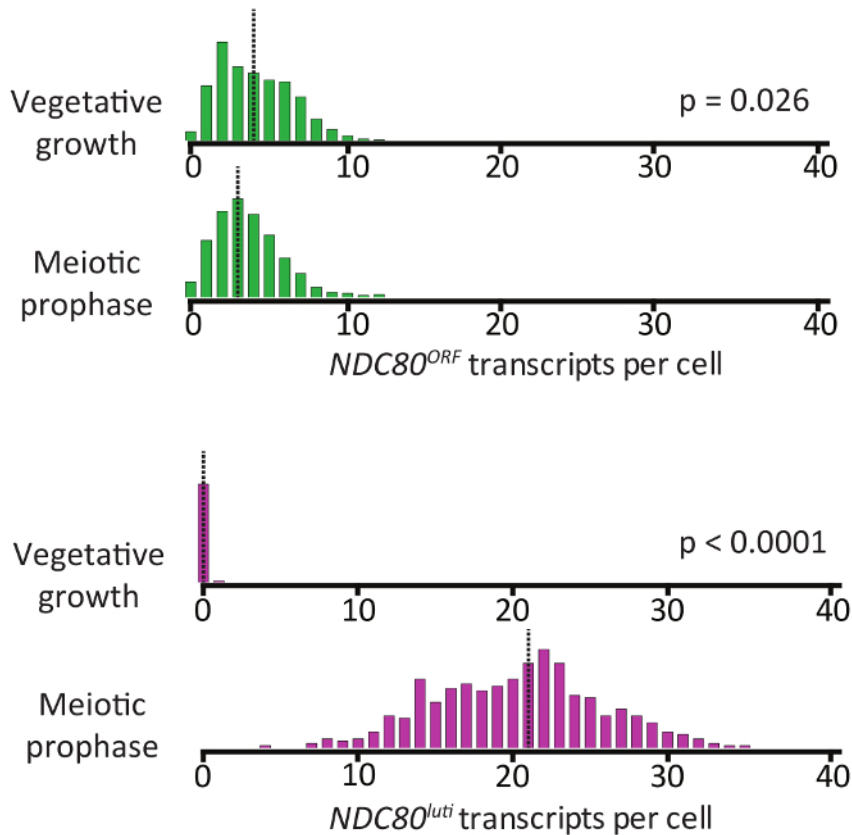
**Figure 4: Quality assessment of the smFISH data generated using the probes designed for *NDC80<sup>uti</sup>* and *NDC80<sup>ORF</sup>* mRNAs.** The Q 670 probes (shown in green) hybridize to the common region shared between *NDC80<sup>uti</sup>* and *NDC80<sup>ORF</sup>* mRNAs, whereas the CF 590 probes (shown in magenta) hybridize to the unique 5' region of *NDC80<sup>uti</sup>*. (A) Representative smFISH images of *NDC80<sup>uti</sup>* and *NDC80<sup>ORF</sup>* in meiotic prophase, acquired under optimized versus suboptimal conditions. Strains UB8144 (optimized condition) and UB1337 (suboptimal condition) were induced to undergo meiosis and fixed during meiotic prophase. These two strains harbor different synchronization systems to induce meiosis, but the use of either system does not affect smFISH quality (data not shown). In the suboptimal condition, cells were fixed at room temperature for 20 min (no overnight fixation), digested with zymolyase without VRC supplemented, and hybridized in a lower concentration of VRC. Images are shown as the maximum-intensity projections of z-stacks. DNA was stained with DAPI (shown in blue). Scale bar: 5  $\mu$ m. (B) Scatterplots displaying the signal-to-noise ratio (SNR) and the signal of each smFISH spot detected in the field of view presented in **Figure 4A**, for either fluorescent channel, as well as for the optimized or suboptimal conditions. In the optimized condition, two populations of the smFISH spots were present. The true smFISH spots were located inside the grey area, separating from the background signal. In the suboptimal condition, individual mRNAs were hard to distinguish by eye, and the separation between the true spots and background after running the spot detection software was less obvious. [Please click here to view a larger version of this figure.](#)



**Figure 5: Expression of *NDC80*<sup>lut1</sup> and *NDC80*<sup>ORF</sup> transcripts are temporally controlled.** (A) Representative smFISH images of *NDC80*<sup>lut1</sup> and *NDC80*<sup>ORF</sup> during vegetative growth and meiosis. Vegetative samples were taken when cells (UB8144) were growing exponentially in nutrient rich medium. Meiotic prophase samples were taken 6 hours after cells (UB8144) were transferred to sporulation medium, a time when these cells were arrested in meiotic prophase. The Q 670 probes (shown in green) hybridize to the common region shared between *NDC80*<sup>lut1</sup> and *NDC80*<sup>ORF</sup> mRNAs, whereas the CF 590 probes (shown in magenta) hybridize to the unique 5' region of *NDC80*<sup>lut1</sup>. DNA was stained with DAPI (shown in blue). Each cell was staged by its Zip1-GFP signal, a marker for meiotic prophase. Our smFISH protocol preserves strong GFP signal without further modification. Vegetative growth: Zip1-GFP negative. Meiotic prophase: Zip1-GFP positive. Images are shown as the maximum-intensity projections of z-stacks. Scale bar: 5  $\mu$ m. This figure is modified from **Figure 2C** of Chen *et al.*<sup>16</sup>. (B) *NDC80*<sup>ORF</sup>, *NDC80*<sup>lut1</sup>, and Ndc80 protein (Ndc80p) level during meiosis (UB4074). *NDC80*<sup>lut1</sup> and *NDC80*<sup>ORF</sup> levels were determined by northern blot, and Ndc80p level was determined by anti-V5 immunoblot at the indicated time points. Hxk1p, loading control for immunoblot. As strain UB8144, this strain also harbors the *pGAL-NDT80 GAL4-ER* synchronization system<sup>19,20</sup>. Cells were transferred to sporulation medium at 0 hour and released from pachytene arrest by  $\beta$ -estradiol addition 6 hours later. The *NDC80*<sup>lut1</sup> transcript was robustly detected in S/meiotic prophase, whereas the *NDC80*<sup>ORF</sup> transcripts was predominantly present prior to meiotic entry (0 hour) and during the meiotic divisions (7-9 hours). This figure is modified from **Figure 6J** of Chen *et al.*<sup>16</sup> [Please click here to view a larger version of this figure.](#)



**Figure 6: Bootstrap analysis performed for the meiotic prophase samples shown in Figure 5.** All of the quantified cells were pooled, and a given number ( $n$ ) of cells were randomly sampled 500 times. The mean and 95% confidence interval were calculated for the fraction of paired and unpaired mRNA per cell. These data were plotted for each choice of the number  $n$  (Inset). The plateau in variance was visualized by plotting the sample variance divided by the mean, as a function of the number of cells ( $n$ ) sampled. The total number of cells measured (437 cells) greatly exceeded the number at which the error plateaued (~60 cells), indicating that additional data would not improve the confidence in the measurements. [Please click here to view a larger version of this figure.](#)



**Figure 7: Quantification of the smFISH data shown in Figure 5, graphed as the relative frequency histograms of the cells with a given number of  $NDC80^{luti}$  and  $NDC80^{ORF}$  transcripts per cell, using the data pooled from three independent experiments.** The dashed line indicates the median number of  $NDC80^{luti}$  and  $NDC80^{ORF}$  transcripts per cell. Each histogram was normalized so that the maximum bin height was the same across all of the histograms. A total number of 637 cells were analyzed for vegetative growth and 437 for meiotic prophase. Two-tailed Wilcoxon Rank Sum test was performed for  $NDC80^{luti}$  and  $NDC80^{ORF}$ , respectively, comparing vegetative and meiotic prophase samples. This figure is modified from **Figure 2D** of Chen *et al.*<sup>16</sup> [Please click here to view a larger version of this figure.](#)

<b>Buffer B (1 L stock: 1.2 M sorbitol; 0.1 M potassium phosphate buffer, pH 7.5)</b>	1x
Nuclease-free water	500 mL
Sorbitol	218.6 g
KH <sub>2</sub> PO <sub>4</sub>	2.18 g
K <sub>2</sub> HPO <sub>4</sub>	14.62 g
Nuclease-free water	Bring to 1 L final volume
*Store at 4 °C in 50-mL aliquots after filter sterilizing	
<b>Zymolyase 100T (10 mg/mL)</b>	
*Store at -20 °C in aliquots	
Dissolve 10 mg zymolyase powder in 1 mL MilliQ water	
<b>E. coli tRNA (10 mg/mL)</b>	
*Store at -20 °C in aliquots	
Dissolve 10 mg tRNA powder in 1 mL MilliQ water	
<b>70% ethanol (50 mL)</b>	1x (mL)
Pure ethanol	35
Nuclease-free water	15
*Store at room temperature	
<b>Hybridization Buffer (10 mL)</b>	1x (mL)
50% Dextran sulfate	2
E. coli tRNA (10 mg/mL)	1
200 mM Vanadyl ribonucleoside complex (VRC)	0.1
BSA, 50 mg/mL	0.04
20x SSC	1
Formamide	1
Nuclease-free water	4.86
*Store at -20 °C in 250-μL or 500-μL aliquots	
<b>10% formamide wash buffer (10 mL)</b>	1x (mL)
Formamide at room temperature	1
20x SSC	1
Nuclease-free water	8
*make fresh, vortex for 20-30s to mix	
<b>10% glucose solution</b>	
*Store at 4 °C in aliquots after filter sterilizing	
Dissolve 1 g of glucose in 10 mL of nuclease-free water	
<b>Glucose oxidase</b>	
*Store at -20 °C in aliquots	
Dissolve 3.7 mg glucose oxidase in 1 mL of 50 mM NaOAc, pH 5	
<b>Anti-Bleach (GLOX) Buffer, without enzymes (1 mL)</b>	1x (μL)
10% glucose in nuclease-free water	40
1 M Tris, pH 8.0	10
20x SSC	100
Nuclease-free water	850
*make fresh, can also store aliquots at 4 °C	
<b>Anti-Bleach (GLOX) Buffer, with enzymes (50 μL)</b>	1x (μL)
Catalase (vortex mildly, it settles easily)	0.5

Glucose oxidase	0.5
100 mM Trolox (dissolved in ethanol)	1
GLOX buffer	50
*make fresh every time, prepare on ice, can be stored at 4 °C for 2-3 hours	

**Table 1. Buffers and media**

## Discussion

The protocol presented here is derived from other published smFISH protocols<sup>2,3,21,22,23</sup>, and is specifically optimized for the yeast strain background SK1. The parameters optimized included the cell number, fixation duration, centrifugation speed and duration, digestion duration, digestion buffer, probe concentration, and hybridization buffer. Other parameters such as hybridization temperature and duration were not optimized. In this section, we share a few notes that would help adapt this protocol to any yeast strain background and the growth conditions of interest.

The cell wall of budding yeast is one major challenge against obtaining high-quality smFISH images in budding yeast because the cell wall prevents probe penetration. Incomplete digestion of the cell wall by zymolyase leads to inefficient hybridization of the probes and high cell-to-cell variability in signal. However, over-digestion can make cells too fragile, leading to significant cell loss during the washing steps and cell bursting during slide preparation for imaging. Thus, optimizing the duration of digestion is crucial. We recommend conducting a pilot smFISH experiment by digesting the same sample for different amounts of time. In our case, we obtained the best smFISH data if we stopped the digestion when ~80% of cells became non-refractive. Typically, for the same growth condition, different genetic mutant strains can be digested with similar timing. However, the duration of digestion differs when compared among different growth conditions and different stages of meiosis in budding yeast. Once the timing is determined, the quality of smFISH is reproducible. Note that for mutant strains with defective cell wall synthesis and/or composition, digestion may be completed in less than 15 min. Use of different batches of zymolyase may also slightly change the digestion timing.

Optimal probe concentration is needed to achieve a high signal-to-noise ratio. We designed and purchased our smFISH probes commercially. Good probes (often 20-mers) should have a percentage of GC ranging from 35% to 45%, a minimum spacing of 2 base pairs between probes, and low cross-reactivity. We used a web-based probe designer from the manufacturer to generate a list of probes, and if there were enough probes to choose from, we would use the BLAST algorithm in the Saccharomyces Genome Database to eliminate the probes with more than 17 base pairs overlapped with other genomic regions. We chose the more photostable fluorescent dye (CAL Fluor 590) for the probe set that anneals to the unique region of *NDC80<sup>uti</sup>* (CF 590) because in this set, the number of probes that satisfied all of the above criteria (20 probes) was lower than the minimum number recommended by the manufacturer (~25 probes). After reconstituting the probe solution following the manufacturer's instructions, we made a 1:10 dilution of the stock solution and stored the diluted solution in 5-μL aliquots at -20 °C. Each aliquot was used only one time. For optimization, one should make serial dilutions from the 1:10 diluted solution and test which concentration yields the best signal-to-noise ratio. We recommend making a dilution series of 1:250, 1:500, 1:1000, and 1:2000 from the original stock. In our case, a 1:500 dilution factor resulted in the best signal-to-noise ratio, for both the CF 590 and Q 670 probe sets.

Optimal fixation time is also critical for successful smFISH in budding yeast. We found that overnight fixation at 4 °C, rather than fixing at room temperature for 20 min, significantly improved the consistency and quality of the smFISH results for meiotic samples. Although we have not tested how overnight fixation might impact vegetative samples, fixation at room temperature worked well for this growth condition. Thus, to optimize the smFISH protocol for new strains or growth conditions, we recommend starting with short fixation time at room temperature and increasing the fixation time if high variability of signal is encountered.

Compared with other published protocols<sup>3,22,23</sup>, our protocol uses the RNase inhibitor VRC during digestion and a higher concentration of VRC during hybridization. Addition of VRC in these two steps improved the consistency of the smFISH results, possibly by better preserving RNA molecules against nuclease activity, which may be introduced by the zymolyase mix (the enzyme is purified from crude extracts and may contain RNase contaminants). Thus, we recommend using the amount of VRC as listed in our protocol or even higher concentrations of VRC for optimization.

A significant portion of the cells can be lost during the washing steps in both Buffer B and the formamide wash buffer. To reduce cell loss, one could increase the centrifugation speed. In our case, using a high speed (21,000 x g) to pellet our samples during the Buffer B washes significantly reduced cell loss. However, cells become very fragile after zymolyase digestion, so changing centrifugation speed is not recommended. Instead, we suggest using the low-adhesion tubes from USA Scientific, which greatly help pellet the cells during the washes in the formamide wash buffer. Overall, our protocol can consistently generate a monolayer of cells dense enough for efficient imaging. Typically, 7 fields of view should yield >130 cells suitable for quantification.

Lastly, it is important to determine the optimal parameters for and the outputs needed from image analysis. To detect smFISH spots, published analysis programs commonly filter the raw images using Gaussian kernel to remove background signal, and ask the users to determine the signal-to-noise ratio to use for each set of images<sup>2,17</sup>. Unfortunately, there is not currently a single standard by which the proper parameters are determined, and so some empirical testing of these different settings is necessary. To set the parameters needed for each of these steps, one needs to iteratively input different sets of parameters and check how well the results obtained from each set matches that from manual counting in a few representative cells. Once one set of parameters is found, it can be used for most of the images obtained despite different growth conditions and genetic backgrounds.

In addition, one can test if maximum-intensity projection of the smFISH images can be performed prior to quantification<sup>22</sup>. This step simplifies the spot detection algorithm and significantly reduces imaging time, though at the cost of some potentially useful information about individual spots,

such as their subcellular localization. In our case, the number of mRNA molecules produced by the *NDC80* gene was low enough that the spots were well separated after this processing (not uncommon for transcripts in budding yeast<sup>3,7</sup>). In cases where colocalization analysis is critical, the analysis pipeline needs to determine the location of each smFISH spot in each channel to assess colocalization. Depending on the specific questions being asked, other information such as the intensity of each spot may also need to be extracted from the pipeline for further analysis. Optimizing the key steps in the protocol and the image analysis pipeline is crucial in obtaining high quality of smFISH data to study the question of interest.

## Disclosures

The authors have no conflict of interest to disclose.

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